EFFECT OF BIOLOGICAL SEX AND AGE ON UNIVERSAL INFLUENZA VACCINE-INDUCED IMMUNITY IN MICE

By

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ABSTRACT

BACKGROUND:

Human data and murine studies demonstrate that biological sex and age impact inactivated influenza vaccine (IIV)-induced immunity, wherein the antibodies are directed towards Influenza virus glycoprotein hemagglutinin (HA) head. Post IIV in mice, females of reproductive age generated greater antibody responses leading to better protection than males. Whereas, in aged mice, where the immunity is waning, the sex-differences are lost and are associated with reduced concentrations of sex-steroids. Whether universal influenza vaccines (UIV) producing antibodies towards the highly conserved stalk region of HA protein are also influenced by biological sex and age has not been studied. We hypothesized that age-associated sex differences to influenza vaccine response will be observed regardless of target region of antibodies.

METHODS:

We used the chimeric hemagglutinin (cHA)-based UIV, which produces antibodies towards the stalk region of the HA protein. We vaccinated adults and aged C57BL6 mice using a prime-boost-boost strategy with a live-attenuated influenza B virus containing chimeric H9/1, recombinant H11/1 and H12/1 protein, respectively. We evaluated the antibody responses of stalk-specific IgG, IgG isotypes and avidity against H1 stalk and cross-reactive IgG response against H9N2, H6N3, H5N1 and H3N2 viruses. We challenged the mice with H1N1 virus to monitor vaccine-induced protection. We also passively transferred antibodies from adults to their sex-matched aged counterparts to evaluate protection.

RESULTS:

Post-vaccination antibody analysis demonstrated that adult mice developed higher quantity and quality of antibody than aged mice. Adult mice had greater stalk-specific IgG titers against multiple subtypes of group 1 influenza A viruses and had greater antibody avidity, regardless of sex. The age-associated decline in immunity was sex-specific, where aging significantly reduced antibody responses in female but not male mice. Post-challenge with H1N1 virus, aged females suffered greater morbidity and had higher viral titers than age-matched males and younger females. Passive transfer of serum from sex-matched adult mice to aged mice was not sufficient to protect aged mice from severe outcomes following infection.

CONCLUSION:

In conclusion, these findings suggest a fundamental difference between males and females during aging. Hence biological sex and age should be considered in preclinical and clinical studies involving all influenza vaccine platforms.

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ABBREVIATIONS

ACIP	Advisory Committee on Immunization Practices
ASO3	Adjuvant System 03
ATCC	American Type Cell Culture
BSL	Biosafety Level
CDC	Centers for Disease Control and Prevention
cHA	Chimeric- Hemagglutinin
DMEM	Dulbecco's modified Eagles Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
HA	Hemagglutinin
HAI	Hemagglutinin Inhibition Assay
HD-IIV	High Dose Inactivated Influenza Virus
HIV	Human Immunodeficiency Virus
IAV	Influenza A virus
IgG/G1/G2c	Immunoglobulin G/G1/G2c
IIV	Inactivated Influenza Vaccine
IL	Interleukin
IM	Intra-muscular
IN	Intra-nasal
IP	Intra-Peritoneal
LAIV	Live Attenuated Influenza Vaccine

LD_{50}	Lethal Dose 50
M2	Membrane Protein 2
M1	Matrix protein 1
MDCK	Madin Darby Canine Kidney Cells
MOI	Multiplicity of Infection
NA	Neuraminidase
NIH	National Institute of Health
NLS	Nuclear Localization Signal
NP	Nucleoprotein
PBS	Phosphate Buffer Saline
PCV	Pneumococcal conjugate vaccine
PFU	Plaque Forming Units
QIV	Quadrivalent Influenza Vaccine
RNP	Ribonuclear Proteins
RPMI	Roswell Park Memorial Institute
SARS	Severe acute respiratory syndrome
SD-IIV	Standard Dose Inactivated Influenza Virus
ssRNA	Single stranded Ribonucleic Acid
TCID	Tissue Culture Infectious Dose
TIV	Trivalent Influenza Vaccine
TLR	Toll like Receptor
UIV	Universal influenza vaccine
WHO	World Health Organization

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INTRODUCTION

Influenza virus biology:

Influenza viruses (family *Orthomyxoviridae*) are segmented, negative-strand RNA viruses requiring an RNA dependent RNA polymerase of viral origin in order to replicate inside the host cell (Bouvier & Palese, 2018). There are three genera of the virus, influenza A, B, and C, identified and known to infect humans. In 2011, a novel influenza virus was identified which was named in 2016 as influenza D virus by the International Committee of Taxonomy of Virus (Ferguson et al., 2016), due to its genetic distinction and inability to reassort with influenza C virus (Su et al., 2017). Since it is a negative sense virus, the RNA in its crude form is not infectious and must be converted to positive-sense RNA in order to be translated (Bouvier & Palese, 2018). The influenza A and B viruses each comprise of eight segments of these negative sense, single-stranded viral RNA. Nomenclature of these viruses, mainly influenza A viruses (IAVs) is based on the subtype of the virus surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Gene segments 4 and 6, respectively, code for HA and NA genes (Bouvier & Palese, 2018).

Influenza viruses recognize N acetylneuraminic (sialic) acid residues on the host cell surface. The carbon 2 of sialic acid can bond with either carbon 3 or carbon 6 of galactose, leading to the formation of alpha 2,3 or alpha 2,6 linkages. These sialic acid moieties are recognized by HA spikes present on the virus. Human influenza viruses can attach to the 2,6 alpha sialic acid residues present in the upper respiratory epithelial cells whereas avian influenza viruses attach to the 2,3 sialic acid residues present on the gut

epithelium of aquatic birds. The 2,3 sialic residues however are also present in the lower respiratory region, mainly on alveolar cells. What makes influenza a respiratory human pathogen is the presence of alpha 2,6 sialic acid residues on the human tracheal epithelial cells (Davis, 2014). The 2,3 sialic acid residues are mainly present in the gut epithelium of aquatic birds, making them a reservoir of these viruses (Medina & García-Sastre, 2011). One of the factors for transmission of avian influenza viruses in humans, as recently observed for H5N1 in 2019 in Nepal (WHO: Information on Avian Influenza A (H5N1) Identified in Human in Nepal), is the presence of these 2,3 sialic acid residues in the lower respiratory epithelium of humans, although in less abundance. Attachment of these exotic influenza viruses to the receptors in humans and subsequent multiplication can cause severe damage due to not having encountered the virus before thereby having no adequate preexisting immunity. The HA region of the virus contains sialic acid receptor binding sites. When the virus replicates, this HA is cleaved by serine proteases into HA1 and HA2, where HA2 mediates the fusion of the virus with the cell membrane. Following the attachment, the virus is endocytosed, and in presence of low pH, two events are observed, first is a conformational change in the HA which exposes its fusion peptide leading to merger of the viral envelope with the endosome membrane. This leads to release of the viral ribonuclear proteins (RNPs) into the cytoplasm (Bouvier & Palese, 2018). Second is the pumping up of hydrogen ions (H^+) from the acidic endosomal environment into the virus through membrane protein 2 (M2) ion channel, which disrupts all the protein-protein interaction allowing RNPs to be released into the cytoplasmic environment. Once released into the cytoplasmic environment, these RNPs travel to the nucleus through the nuclear localization signal (NLS). The nucleus is the site where RNA

synthesis occurs for both the viral RNAs and the mRNA for protein synthesis (Fukuyama & Kawaoka, 2011).

Of the three genera infecting humans, Influenza A viruses (IAVs) cause most seasonal infections and cause all pandemics, to date. The primary natural host species of IAVs include wild aquatic waterfowl and shorebirds (Webster et al., 1992), but cross-species transmission can occur which leads to sustained influenza outbreaks in poultry and mammals, such as humans, pigs, horses, seals and mink (Parrish, Murcia, & Holmes, 2015). Influenza viruses are predominantly respiratory pathogen in humans. They replicate in both the upper respiratory and lower respiratory tract and the replication peaks approximately 48 hours post-inoculation into the nasopharynx and decreases gradually (Taubenberger & Morens, 2008).

Influenza pathogenesis and epidemiology:

Influenza is an acute respiratory disease in humans identified with the onset of high fever, coryza, cough, headache, prostration, malaise, and inflammation of the upper respiratory system (Su et al., 2017). Influenza affects people of all ages, but the prevalence is observed highest in school-age children. The severity of the disease varies within the age, with infants, aged population and people with underlying illnesses suffering the most. Infection with IAVs in people with diabetes mellitus, chronic pulmonary, or cardiac diseases leads to a high risk of progressing to severe complications, including pneumonia, bronchitis and death in some cases (Taubenberger & Morens, 2008).

The major reason why influenza is an annual threat, an economic burden, and needs millions of dollars to be invested in vaccine production is due to a phenomenon called antigenic drift observed in the virus. Antigenic drift refers to a mechanism through which variations are observed in the seasonal influenza viruses in the antibody binding sites to avoid neutralization by the immune response (Chen & Deng, 2009). The mutation rate of HA and NA of Influenza A is estimated to be at $6.7 * 10^{-3}$ nucleotide substitution (Lofgren et al., 2007). These mutations essentially make the antibodies generated against the prior season's viruses ineffective, rendering an advantage to the virus to spread and infect.

Influenza A viruses have been associated with causing multiple pandemics over the last century, killing millions of people around the world (Kilbourne, 2006), with pandemic IAVs caused by an antigenic shift in the circulating virus (Manley, 2013). Antigenic shift refers to the evolution of novel strains of influenza through reassortment of its gene segments with an exotic influenza virus such as an avian or zoonotic influenza virus. Zoonotic infections, in some cases, such as Ebola and Hantavirus infections, may be sporadic and severely pathogenic, rendering humans as a dead-end host. Whereas, some infections with a zoonotic pathogen can lead to it becoming stable for replication and transmission in the human population, such as observed in HIV and SARS (Manley, 2013). In the case of the influenza 2009 H1N1 pandemic, the virus had partial elements from the human virus and partial from a zoonotic influenza virus and was able to replicate and transmit disease in the human population (Christman et al., 2011). Since the zoonotic elements of the virus have been "unseen" by the human immune system, they can be severely detrimental to human health.

In the US, the Centers for Disease Control and Prevention (CDC) has estimated around 9.3 to 49 million influenza-related illnesses, around 140,000 to 960,000 hospitalizations, and around 12,000 to 79,000 deaths annually in the US alone (CDC: Estimated Influenza Illnesses, 2017). According to the World Health Organization (WHO), the seasonal influenza epidemics result in 3 to 5 million cases of severe illnesses and around 290,000 to 650,000 respiratory deaths, mainly affecting aged population (>=65 years of age) in industrialized countries (WHO: Seasonal Influenza, 2014). The exact statistics and extent of seasonal influenza are not known in developing countries but is expected to affect children under five years of age the most (Lafond et al., 2016). IAVs have also been associated with causing multiple pandemics over the last century, killing millions of people around the world (Kilbourne, 2006). Such pandemics occurred during the years 1918, 1957, 1968 and 2009 **(Figure 1)** (Christman et al., 2011). Antigenic drifts of influenza viruses are responsible for the seasonal influenza epidemics, while the antigenic shift is responsible for pandemics.

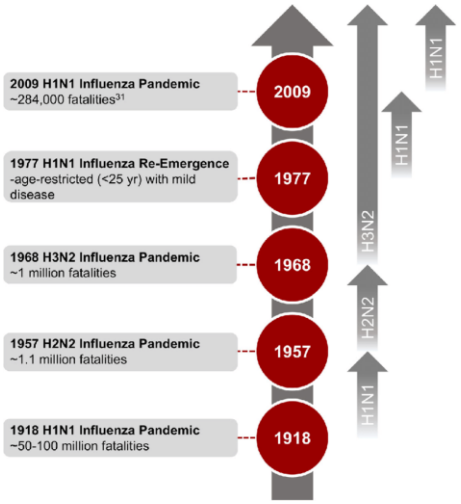


Figure 1: Influenza pandemics in the last 100 years (Nickol & Kindrachuk, 2019)

Influenza control measures: vaccination and prevention

The best way to prevent the spread of influenza viruses is through vaccinations (WHO, 2014). In children, the Inactivated influenza vaccine (IIV) is an efficient and cost-effective intervention, despite some of its limitations (Salleras et al., 2013). The CDC estimated that influenza vaccines prevented 7 million influenza illnesses, around 100,000 hospitalizations, and 8000 deaths in the 2018-2019 season (CDC 2018). The majority of deaths averted by influenza vaccines in the previous decade were in aged people and children younger than five years of age (Foppa et al., 2015). The currently available seasonal vaccines, however, must be reformulated every year, depending on the circulating influenza strain. Thus, the effectiveness of these vaccines depends on if the circulating strains match the selected vaccine strains. Reformulation takes place twice each year for the northern and southern hemisphere and 7-8 months before the influenza season selection of vaccine strains is finalized to begin the vaccine production (Houser and Subbarao, 2015).

There are currently three types of vaccines for influenza that are licensed and available in the US: 1) the inactivated influenza vaccine, 2) live attenuated influenza vaccines, and 3) recombinant influenza vaccines (Houser and Subbarao, 2015). All the three licensed vaccines are multivalent and comprise components of both influenza A and B viruses.

The **inactivated influenza vaccines** (IIVs) are available as trivalent (TIV), composed of two subtypes of Influenza A (H1N1 and H3N2) and one strain of Influenza B virus or as quadrivalent (QIV), having two subtypes of influenza A virus and two lineages (Victoria and Yamagata) of influenza B virus. The commercially available

inactivated vaccines such as Fluarix, FluLaval, Flucelvax are quadrivalent, and Afluria and Fluzone are trivalent vaccines. These are split virion/subunit vaccines that contain 15 μg of the purified HA protein, unadjuvanted, from egg grown virus, delivered intramuscularly (CDC, 2018).

Twice every year, the WHO consults with its collaborating centers for reference and research on circulating influenza virus strains and makes recommendations on the composition of the year's influenza vaccine, based on the surveillance and laboratory data (Stöhr, Bucher, Colgate, & Wood, 2012). The seasonal IIVs are also available as both high dose and adjuvanted vaccines for aged individuals and have shown to increase the antibody response and decrease influenza-related illnesses in this population (DiazGranados et al., 2014). The high dose vaccine contains four times the amount of antigen as a regular influenza shot and the adjuvanted influenza vaccine, commercially available as Fluad, contains the MF59 adjuvant, which has shown to stimulate CD4 memory T cell response (Falsey et al., 2009).

The **live-attenuated influenza virus (LAIV) vaccines** are comprised of the same influenza strains present in the QIV but as a live virus delivered intranasally. The live virus has specific mutations which make it temperature-sensitive and hence can replicate in the cooler temperature of the nasal cavity, as opposed to the higher temperature of the lower respiratory tract, restricting its replication there (Houser and Subbarao, 2015). LAIV results in the production of strain-specific IgG responses, as observed in response to IIV, but also stimulates mucosal IgA and T cell responses. LAIV is also effective in protecting and generating a response against some strains of antigenically drifted influenza viruses (Coelingh, Luke, Jin, & Talaat, 2014). In the United States, however,

the Advisory Committee on Immunization Practices (ACIP) voted on discontinuation of the LAIV for the 2016-2017 season. The preliminary data of LAIV effectiveness in children between 2 to 17 years of age showed exceptionally low effectiveness of 3% as compared to 63% effectiveness in its alternative method, IIV (CDC, 2017)

The third type of influenza vaccine is a **recombinant HA protein vaccine**, commercially available as FluBlok. This recombinant HA protein vaccine is produced in a baculovirus vector expressing the HA protein. Despite the limitation of its short shelf life, it is recommended for vaccinating adults above 18 years of age and is ideal for people with egg allergy (Houser and Subbarao, 2015). FluBlok contains three times the amount of HA present in TIV and has shown to induce a higher HAI antibody response compared to TIV. Hence it can be a good option for populations at risk for influenza (M. M. J. Cox, Patriarca, & Treanor, 2008).

Anti-viral treatments:

Vaccinations are currently the best way to minimize the risk and transmission of influenza. Apart from those, there are anti-viral treatments available as recommendations for patients with confirmed cases of influenza who are hospitalized, have a severe or progressive illness or are at a high risk of influenza complications such as children under the age of 2 years, older individuals, people with co-morbidities and pregnant females. There are multiple drugs available targeting different influenza virus proteins. Neuraminidase Inhibitors, endonuclease inhibitors and drugs targeting the M2 ion channel protein (CDC: Antiviral Drugs for Seasonal Influenza).

Limitations of current seasonal influenza virus vaccines:

Dependence on embryonated eggs: The dependence of vaccines on production in chicken eggs results in the insertion of egg-adaptation mutations that are not present in circulating strains (Yang et al., 2019). Also, production takes an extended period, which can cause delays or limits in available doses if some selected viruses do not grow well in chicken eggs (Rajaram et al., 2020). In the case of a pandemic, when the demand is immediate and higher, the use of egg for vaccine production would be an obstacle, especially if the pathogen infects poultry (Houser and Subbarao, 2015) (Hannoun, 2013).

Lengthy timeline for vaccine production: Vaccine production for influenza includes multiple steps, the majority of which have to be done before the influenza season. This includes starting from evaluating the surveillance data, selecting the vaccine strains, virus amplification, vaccine preparation to the packaging of the vaccine. This process takes a lot of time and effort, like every other vaccine, but especially becomes a prolonged economic burden when it must be repeated annually (Smith et al., 2014).

Annual vaccination: The major limitation to influenza vaccines is the annual need for vaccination observed due to a decline in vaccine-specific antibodies along with the annual variation in the virus surface glycoprotein. Several researchers are working on developing vaccines targeting the conserved HA stalk region, M2 ion channel and NP protein (Krammer & Palese, 2013). Most of the vaccines against other infectious diseases are required or recommended once or twice in a lifetime, whereas vaccination against influenza becomes a burden due to its annual need. The seasonal vaccine coverage for influenza during the 2018-2019 season was seen to be less than 50% in individuals aged above 18 years and children less than six years of age, depicting

peoples' lack of interest in annual vaccination (CDC: Early-Season Flu Vaccination Coverage–United States, November 2018). United States is one of the countries that have achieved the WHO's recommended goal of increasing influenza vaccine coverage up to 50% by 2006 for individuals aged 65 years and older, which have coverage of 71.5% as of 2017. This has not, however, resulted in a corresponding decrease in the morbidity and mortality in this aged population, indicating the incompetency of the available vaccines in the elderly population, which comprise of almost 15% of the total US population (United States Census Bureau, 2019).

Ineffective against novel strains: The current influenza vaccines are enough for protection against seasonal circulating strains but are essentially ineffective against a novel strain of influenza virus. With the close contact of humans with poultry and animals, such as pigs, the potential for recombination of zoonotic influenza with human influenza becomes higher, as previously observed in the multiple pandemics (Taubenberger & Kash, 2010). Both IIVs and LAIVs have been tested as potential vaccine candidates for influenza pandemics and shown to display low immunogenicity and need for multiple vaccinations (Baz & Luke, 2013). A better solution would be an influenza vaccine targeting the conserved sites of influenza viral proteins and providing long-term protection against multiple influenza subtypes.

Immunosenescence in the elderly: Influenza most severely affects the aged population, i.e., individuals above 65 years of age, due to progressive decline in immune response observed in this population, termed as immunosenescence (Aw, Silva, & Palmer, 2007). Even in the circumstances of the vaccine strain matching the circulating influenza virus strain the IIV, the efficacy is observed to be 70-90% in young adults but

ranges from 17 to 51% in individuals over 65 years of age and further declines in those over 70 (Goodwin, Viboud, & Simonsen, 2006). The efficacy of the IIVs is seen to be waning in elder individuals. Immunization leads to the production of vaccine-specific B cells, which differentiate into vaccine-specific plasmablasts in the germinal centers (GCs), and by 6-8 days, they leave the GCs and enter the blood circulation. ELISPOT analysis of vaccine-specific plasmablasts at 7 days post-vaccination revealed a decrease in their number and the concentration of Plasmablast-derived polyclonal antibodies (PPAbs) in the elderly as compared to the young adults (R. J. Cox et al., 1994) (Sasaki et al., 2011). A recent meta-analysis study comparing the relative vaccine efficacy of high dose inactivated trivalent influenza vaccine (HD-IIV3), and standard-dose inactivated trivalent influenza vaccine (SD-IIV3) demonstrated that HD-IIV3 exhibited better protection against influenza-like illnesses than SD-IIV3 with a relative vaccine efficacy of 19.5% (Fisman, Agrawal, & Leder, 2002). The current seasonal influenza vaccines have a higher dose recommended for the elderly to enhance the immunogenicity of the vaccines in this population. The specific chain of mechanisms governing this lower efficacy of IVs in the elderly population is, however, still under investigation.

Absence of an accurate correlate of protection: The hemagglutinin inhibition (HAI) assay is presently considered as the standard measure of antibody titer induced by vaccination and is accepted as the correlate of protection against influenza (Houser and Subbarao, 2015). A titer of 1:40 is the threshold, beyond which approximately 50% of the population is considered protected from influenza infection. However, this assay does not consider the T cell response generated by the body after vaccination and there is poor reproducibility in the HAI results from one laboratory to another (Zacour et al., 2016).

The HAI titer threshold for protection is not consistent for all age groups as well. (Weiskopf, Weinberger, & Grubeck-Loebenstein, 2009) (Davies & Grilli, 1989)

Universal influenza vaccines:

As per the limitations discussed above, there is a need for an improved influenza vaccine, including the consideration of universal influenza vaccines. "Universal", as such, is a misleading term, but the National Institute of Health (NIH) has some guidelines for a vaccine to be considered as a universal influenza vaccine. It includes that the vaccine should be protective for more than a year, should protect against group 1 and group 2 influenza viruses, should be at least 75% effective, and must be safe for all age groups (NIH: Universal Influenza Vaccine Research). In the case of seasonal IIVs, the major antibody target is the immunodominant HA head. This head region is highly mutating, making the current season's vaccine ineffective next season (Houser and Subbarao, 2015). The membrane-proximal stalk region of the HA is an immune-subdominant, highly conserved region. It has been challenging to develop a vaccine against the conserved stalk region due to its immune-subdominant nature and the fragile nature of its epitopes (Krammer & Palese, 2013).

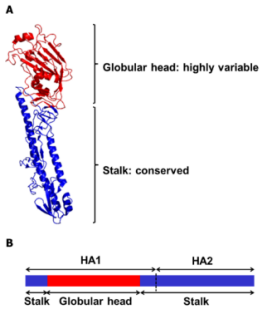


Figure 2: Ribbon diagram and schematic representation of the influenza HA protein (Y. Jang & Seong, 2014)

The predominant immune response generated in response to current influenza vaccines is the neutralizing antibody response (humoral) against the HA protein head. Contrarily, the universal influenza vaccines currently in development are focusing on generating a broader immune response comprised of humoral, cellular, and innate responses (Sasaki et al., 2008). Currently, there are several universal influenza vaccine platforms in development. The principal targets for universal influenza vaccine development include the HA stalk region, headless HAs, nucleoprotein (NP), and M2 ion channel protein (Rao et al., 2010). NP protein is involved in encapsidation of the viral genome, which forms a ribonucleoprotein particle for transcription and packaging. NP also interacts with other influenza viral proteins, such as PB1, PB2, M1 and cellular proteins such as F-actin for transcription control (Portela & Digard, 2002). The M2 protein, on the other hand, is involved in protein translocation and is also the primary target for influenza prophylactic drugs such as amantadine and rimantadine (Pielak & Chou, 2011). The NP protein and M2 ion channel protein have an essential task in virus pathogenesis and are being researched in animal models as a potential universal influenza vaccine candidate for clinical trials (Y. H. Jang & Seong, 2019).

The other two significant approaches to induce a broadly neutralizing antibody response are constructing headless HAs, chimeric HA protein, or mosaic HA protein platform(Krammer & Palese, 2019). The first headless HA constructs were developed in the 1980s, by treating the virus with acid to unmask the HA stalk and with a reducing agent to remove the HA head region (Graves, Schulman, Young, & Palese, 1983). This treatment, however, destroyed the conformational epitopes of the virus. Recently, Yassine et al. reported stable headless HA constructs being developed, which induced

stalk reactive antibodies in mouse models, and further investigation on its efficacy is under investigation (Yassine et al., 2015).

The chimeric hemagglutinin(cHA) based universal influenza vaccine approach combines two elements: 1) Use of chimeric HAs and 2) sequential vaccination. A chimeric hemagglutinin is a recombinant HA protein that combines the use of HA stalk domain from a human influenza virus with "exotic" globular head domains derived from another influenza A virus subtypes (Hai et al., 2012). By repeatedly vaccinating mice with chimeric HAs containing the same stalk and a different head construct for each vaccination leads to the production of enhanced stalk directed response. These stalk specific antibodies are found to be broadly neutralizing and protective against infection with pandemic H1N1, H5N1, and H6N1 viruses in the mouse model (Krammer et al., 2013).

Recently published interim results of the randomized, placebo-controlled, phase 1 clinical trial of the chimeric hemagglutinin universal influenza vaccine assessed the efficacy of three regimens: 1) chimeric H8/1 (H8 head/H1 stalk) hemagglutinin-based live-attenuated vaccine followed by the boost with a non-adjuvanted chimeric H5/1 hemagglutinin-based inactivated vaccine; 2) chimeric H8/1 hemagglutinin-based live attenuated vaccine followed by a boost with an ASO3-adjuvanted chimeric H5/1 hemagglutinin-based inactivated vaccine and; 3) an ASO3-adjuvanted cH8/1 IIV prime followed by an ASO3 adjuvanted cH5/1 IIV boost. They demonstrated that the inactivated regimens induced a sufficient anti-HA stalk antibody response post boost against H2, H9 and H18 recombinant hemagglutinins, pandemic H1N1, and avian-swine H1N1 and H5N8 viruses. The memory cell response, however, declined 28 days post-

second boost, questioning the potential of this vaccine platform as a truly universal influenza vaccine. However, the production of cross-reactive antibodies and lack of safety concerns make it a good candidate for a pandemic vaccine, which can be delivered in cases of future unexpected pandemics (Bernstein et al., 2020).

Host factors affecting vaccine efficacy:

Variations in immune responses to vaccinations have been observed at the population as well as individual level, linked to several cofactors harbored by the vaccinees. In an HIV g120 vaccine clinical trial, race/ethnicity was observed to influence the geometric mean titers of neutralizing antibody responses, with African American participants having 2.6-4.7 times higher GMTs than white participants (Montefiori et al., 2004). Genetic factors such as the highly polymorphic Human Leucocyte Antigen (HLA) system involved in antigen presentation and less polymorphic pathway of Toll-like receptors involved in antigen recognition are involved in varying immune responses observed to vaccinations (Kimman, Vandebriel, and Hoebee, 2007). Coexisting conditions and infections impact immune responses to vaccines as well. Underlying helminth infections among cholera patients demonstrated reduced mucosal immune responses to an immunodominant cholera antigen, Cholera toxin B (CTB), a component of an internationally licensed oral cholera vaccine, Dukoral (Baldauf et al., 2015). Aging also has a notable impact on the outcome, susceptibility, spread of infections, and correspondingly also impacts one of the infectious disease prevention strategies, vaccinations. The seasonal influenza vaccine efficacy is lower in the elderly population (i.e., individuals 65 years and older) than younger adults, with the elderly population accounting for 54% to 70% of seasonal influenza-related hospitalizations and 71% to

85% seasonal influenza-related deaths (Wilhelm, 2018). Hepatitis B vaccine has been linked with eliciting a varying degree of response amongst different age groups, with an elevated risk of nonresponse to Hep B vaccines in older individuals (Fisman et al., 2002). In a Community-Acquired Pneumonia Immunization Trial in Adults (CAPiTA), the efficacy of the pneumococcal conjugate vaccine, PCV13, in preventing vaccine-type community-acquired pneumonia (VT-CAP) and vaccine-type invasive pneumococcal disease (VT-IPD), was shown to decline with increasing age (Van Werkhoven et al., 2015).

One of the factors leading to this trend is "Immunosenescence". Immunosenescence is the age-associated progressive decline in immune response which is characterized with a decline in several lymphoid-biased hematopoietic stem cells, naïve B and T cells, impaired B cell function, thymic involution and many more changes leading to decline of a robust immune response(Simon, Hollander, & McMichael, 2015). These molecular and cellular changes affect the response expected to vaccines and will be discussed further in detail in the context of influenza vaccines in upcoming sections.

Biological sex and gender have also been associated with affecting the influenza virus vaccine acceptance, responses, and overall efficacy (Flanagan et al., 2017). Biological sex, which is defined based on the anatomy of an individual's reproductive organs, secondary sex characteristics, sex-specific hormones, and sex chromosome complement, can affect immunological responses to vaccines (Flanagan et al., 2017). There is a female-biased immune response observed after vaccination with influenza, Hepatitis B, Herpes virus and small-pox vaccine (Taneja, 2018). The primary correlate of protection for vaccines is typically the humoral immune response. This antibody response

has been observed to be higher in females than males post-vaccination in case of seasonal IIVs, yellow fever vaccine, rubella, measles and mumps vaccine, rabies vaccine and dengue vaccines (Klein, Jedlicka, & Pekosz, 2010). Aged males are shown to generate a higher antibody titer to the Td/Tdap vaccine (Bayas et al., 2001). Apart from the impact of biological sex on vaccines, there is also a visible impact gender creates on the rate of acceptance of vaccines, uptake of vaccines and reporting of adverse conditions to vaccinations, where females have shown to report more adverse reactions to vaccines than males (Eilers, Krabbe, & de Melker, 2014). Overall, the nonconformity in vaccine efficacy amongst different sexes and genders calls for a need to consider this host factor as an important element in vaccine clinical trials and hence requires more in-depth research dictating the mechanisms of variance.

Of the multiple factors impacting the vaccine efficacy, this project approached the evaluation of influenza vaccine efficacy specifically through an outlook of biological sex and age.

Impact of biological sex on influenza vaccine-induced immunity

Both the biological sex and gender, as mentioned earlier, has shown to impact the comprehensive response to infections and vaccinations. In the case of influenza infections, WHO has reported the worse outcome of pandemic influenza and avian H5N1 infections to appear in young adult females, pressing the need for considering sex and gender as an essential issue while evaluating outcomes of a disease in a population. The 2009 H1N1 pandemic aftermath showcased a significantly high hospitalization rate in

young adult females (Sex, gender and influenza - World Health Organization). Multiple hypotheses are highlighting the effect of differences in exposure to the virus through behavior or occupation leading to these differences. Nevertheless, biological differences in the sexes also contribute to these differences in outcomes and need further investigation in understanding the molecular drive for these differences.

When adult male and female C57BL/6 mice were inoculated with mouse-adapted H1N1 (i.e., A/Puerto Rico/8/34) or H3N2 (i.e., A/Hong Kong/68) using 5 $\log_{10}$ dilutions to determine the lethal dose 50 (LD_{50}), i.e., the amount of virus required to kill 50% of the test population) for each sex, the LD_{50} for females was seen to be 11-fold lower for H1N1 and four-fold lower for H3N2 than the LD_{50} for males (Lorenzo et al., 2011). Various studies have demonstrated the immune response to influenza vaccines, but a few studies mention the role played by biological sex and its impact on vaccine efficacy. Seasonal IIVs and LAIV have coverage between 40-50% in the adult population in the US (CDC US Flu Vaccination Coverage), hence assessing the impact of host factors such as the biological sex is vital in evaluating vaccine efficacy.

In 2018, Fink et al. demonstrated the impact of biological sex, specifically in the case of influenza vaccine-induced immunity and the possible mechanisms leading to this observation. Their murine studies demonstrated three major results: 1) infection of male and female mice intranasally with low dose 2009 H1N1 virus resulted in greater IgG and neutralizing antibody responses, along with higher total B cell IgA, greater germinal center B cells, larger germinal centers in female mice than the male mice. Females also had greater frequencies of CD8+ T cells, CD8+ central memory cells, and tissue-resident memory T cells in lung tissue as compared to males; 2) vaccination with monovalent IIV,

which protects through antibody-mediated responses, resulted in higher IgG and neutralizing antibody response in female mice along with females showing better virus clearance than males, post-challenge with an H1N1 drift variant virus; 3) epigenetic control of *Tlr7* (which is encoded on the X chromosome) expression in B cells is greater in splenic B cells from vaccinated females than males (Fink et al., 2018). Females have two X chromosomes in their cells, whereas males contain one X and one Y chromosome. In females, one of these X chromosomes is randomly inactivated through X chromosome inactivation (XCI) to balance the dosage of gene expression between the females and males (Schurz et al., 2019). This X chromosome has both innate and adaptive immune system genes, including TLR7 and 8, IRAK1, NF-kB essential modulator (Kawai & Akira, 2006)(Bustamante et al., 2011).

TLR7 is both an X-linked gene and an intracellular receptor that recognizes ssRNA and has been shown to successfully escape the X inactivation (Souyris et al., 2018). TLR7 localizes in the endosomal compartment of the cell and elicits a robust type 1 interferon response upon sensing ssRNA and is essential for the development of germinal centers, somatic hypermutation, and selection of high-affinity B cells (Swiecki & Colonna, 2015). Fink et al., revealed that this TLR7 expression was higher in B cells isolated from vaccinated female mice as compared to the vaccinated male mice, which could explain the better antibody response observed in the female mice (Fink et al., 2018). Human serological data have demonstrated that young adult females (18-45 years old) had higher IL6 response, which is involved in the germinal center formation, antibody production and class switching, than the males, post-vaccination with the TIV (Kopf et al., 1998) (Potluri et al., 2019).

Along with the role of genes, sex hormones have shown to impact the immune response as well (Taneja, 2018). The immune cells, such as neutrophils, mast cells, macrophages, B cells and T cells, have the androgen or estrogen receptor on its surface (Lai et al., 2012). Binding of sex hormones with these receptors leads to downstream changes in signaling cascades, which can further regulate the response of those immune cells (Fish 2008). Estrogen is known to modulate the B cell response and activate the Th2 response after encountering a pathogen (CUTOLO et al., 2006). In the case of testosterone, this male-specific hormone has shown to activate T cell, precisely CD8 T cell response and enhancing Th1 response and production of the anti-inflammatory cytokine IL-10 (Taneja, 2018). A recent paper published by Potluri et al., highlights the role of sex hormones specifically in influenza vaccine induced immunity. In humans, they evaluated the antibody response in adults and aged population post vaccination with the monovalent A/Cal/09 H1N1 vaccine. Adult females had a higher neutralizing seroconversion rate post-vaccination than the males, but not in the case of the aged population, which correlated with higher estradiol concentration in adult females than the aged female and higher testosterone concentration in adult male than aged male. Mouse studies conducted to confirm the consistency of the results obtained in humans demonstrated that the adult female mice had significantly higher neutralizing antibody titers at day post-vaccination 35, and higher IgG titers, IgG2c titers at both 28 and 35 days post-vaccination with the monovalent A/Cal/09 H1N1 vaccine with a prime-boost strategy. When the adult mice were gonadectomized and then vaccinated, the female-

biased immunity observed was no longer detected, confirming the correlation between sex hormones and the vaccine response (Potluri et al., 2019).

Impact of aging on influenza vaccine-induced immunity

Aging is accompanied by a decline in physiological functions in the body, progressing towards age-associated mortality (Flatt, 2012). Multiple theories such as antagonistic pleiotropy theory (AP), mutation accumulation theory, theories involving a reduction in the number of stem cells, senescence resulting from "aging" genes, free radical damage, DNA damage, changes in telomere length and planned cell death have been proposed to explain the phenomenon of aging (Jin, 2010). Defining aging in a way classifies scientists, where some consider aging as a disease, whereas some consider it a natural process. Aging, either as an immanent process or a disease in disguise, has an effect on an individual's immune system and concomitantly on vaccine efficacy.

Older adults are at a higher risk of influenza-related illnesses, with the highest hospitalizations and influenza-related morbidity in individuals greater than 65 years of age than any other age group (CDC: People 65 Years and Older & Influenza). The influenza vaccine has shown to elicit a low antibody response in aged individuals than young adults. One of the solutions to this issue is to increase the dose of the current vaccine formulations. The inactivated seasonal influenza vaccine is available as both high dose and adjuvanted vaccines and has shown to increase the antibody response and decrease the influenza-related illnesses in the aged individuals (CDC: People 65 Years and Older & Influenza). The high dose vaccine contains four times the amount of antigen as a regular influenza shot and the adjuvanted influenza vaccine, commercially available as Fluad, contains the MF59 adjuvant which has shown to stimulate CD4 memory T cell

response. Simonsen L et al., in 2007 published data and a review of multiple influenza vaccine efficacy trials demonstrating a variation in influenza rates in the elderly, which are seen to be rising in synchrony with vaccination rates, majorly showcasing the lack of sufficient protection provided by the seasonal influenza vaccines in the elderly. Yao X et al. published the results of the Beeson project, comparing the immune response post influenza vaccination in frail and non-frail aged. Frailty, a critical geriatric syndrome, was shown to be associated with impairment of TIV vaccine-induced antibody response and an increase in post-vaccination influenza infection (Yao X et al., 2011).

Potluri et al., in 2019, published data determining the immune response post-vaccination with the monovalent 2009 H1N1 vaccine in different human age groups. The serological data showed that the adult females (18-49 years old) had a higher HAI and neutralizing antibody seroconversion than adult males and the aged males and females (65+ years old), 21 days post-vaccination. Their murine influenza vaccination studies demonstrated that post-vaccination with inactivated A/Cal/09 H1N1 influenza vaccine, the adult female mice (2-3 months old) had a significantly higher total IgG, IgG2c and neutralizing antibody response than the adult male and aged female (16-17 months old) mice (Potluri et al., 2019), depicting not just an age difference but also a sex-specific age difference in vaccine-induced immune response, with aged females showing a significant decline in immunity and higher morbidity as compared to adult females.

As mentioned earlier, the IIVs have their primary response towards the HA head region, whereas several of the UIV in development have an antibody response directed towards the HA stalk. Whether these universal influenza vaccines are also influenced by host factors, biological sex and age, has not yet been studied and formed the basis of this

study. To understand this, we focused on evaluating the impact biological sex and age has on chimeric hemagglutinin (cHA) based universal influenza vaccine-induced immunity in the mouse model.

MATERIALS AND METHODS

Animals:

Adult (8-10 weeks) and aged (68-70 weeks) male and female C57BL/6 mice were purchased from Charles River Laboratories or the National Institute on Aging, respectively. All animals were housed 5 per cage. The mice were housed under standard biosafety level (BSL) 2 conditions with a 14:10 light: dark cycle and food and water provided ad libitum. All animals were given 2-4 weeks to acclimate to the housing conditions of Johns Hopkins School of Public Health.

Ethics statement:

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee (MO18H250). This research project utilized mice, which are essential because their immune responses to vaccines mimic those observed in humans. The animal testing for this research is irreplaceable because studying the impact of the vaccination requires studying immune responses in an *in vivo* model that cannot be evaluated through *in vitro* study. Only trained individuals certified by the Johns Hopkins University for Animal Care and Use were allowed to handle the animals. The animals were continuously under supervision and were maintained in a healthy environment.

Cells and Viruses:

Madin Darby Canine Kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco). Each was supplemented with 10% fetal bovine serum (HyClone) and

100 units/ml of penicillin-100 µg/ml of streptomycin (Pen/Strep; Gibco). Dr. Florian Krammer, in Icahn School of Medicine at Mount Sinai, New York, USA, provided the live influenza B virus (B/Yamagata/16/1988 virus; 1.4 x 10^7 PFU/ml) expressing chimeric H9/1 HA protein, cH11/1 recombinant chimeric HA protein and cH12/1 recombinant chimeric HA protein for mice vaccination. H9N2 virus protein (HA and NA from A/Chicken/Hong Kong/G9/1997, backbone from A/PR/8/1934), H6N3 virus protein (HA and NA from A/Swine/Missouri/4296424/2006, backbone from A/PR8/1934) and H5N1 virus protein (HA and NA from A/Vietnam/1204/2004, backbone from A/PR8/1934) for testing the cross-reactive serum anti-stalk IgG antibody responses. Mouse-adapted A/California/04/2009 H1N1virus generated by Dr. Andrew Pekosz, Johns Hopkins Bloomberg School of Public Health, was used for the challenge studies in mice.

Vaccination and Infection:

We utilized a cHA universal influenza vaccine regimen with prime-two boosts (LAIV-IIV-IIV) strategy (Krammer et al., 2013). The first vaccination comprises of a live influenza B virus (B/Yamagata/16/1988 virus; 1.4 x 10^7 PFU/ml), containing a chimeric H9/1 HA protein, delivered intranasally (IN) at a dose of 2.0 x 10^5 PFU in 50µL PBS per mouse. The first vaccination primed the immune response to the HA stalk. The second vaccination, administered 3-weeks later comprised of 10 µg of cH11/1 recombinant chimeric HA protein and10µg of Poly I:C adjuvant in sterile phosphate buffer saline (PBS) that was administered IN and intra-muscularly (IM) at 5µg/dose in 50µL/route. The third vaccination administered 3-weeks later comprised of 10 µg cH12/1 recombinant chimeric HA protein, 10µg of Poly I:C adjuvant and PBS administered IN and IM at 5µg/dose in 50µL/route. This prime- two boost regimen was followed by blood

collection via retro-orbital bleeding to obtain serum from the vaccinated and unvaccinated mice three weeks after the third vaccination. One week after the blood collection, mice were challenged IN with a mouse-adapted A/California/04/09 virus at a dose of 10^2 $TCID_{50}$ in a volume of 30μL under ketamine-xylazine anesthesia (100mg/ml). Subsets of mice were either euthanized at 5 days post-challenge to collect lung tissue for viral titration or were monitored for two weeks for changes in body mass and body temperature. Mice below 30% of weight loss as compared to its weight on the day of infection were discontinued from the study and euthanized.

Anti-Influenza HA stalks Enzyme-Linked Immunosorbent Assay (ELISA):

Enzyme-linked immunosorbent assay (ELISA) plates (Flat bottom, Microlon 96-well high-binding plates manufactured by Greiner Bio-One) were coated with 100 ng of purified virus protein of mouse-adapted H1N1 or H3N2 (A/Hong Kong/1968); 250ng of H9N2 (HA and NA from A/Chicken/Hong Kong/G9/1997, backbone from A/PR/8/1934), H5N1(HA and NA from A/Vietnam/1204/2004, backbone from A/PR8/1934) or H6N3 (HA and NA from A/Swine/Missouri/4296424/2006, backbone from A/PR8/1934) viruses in a carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing with wash buffer (1x PBS + 0.1% Tween-20), the plates were blocked with 10% dry milk in wash buffer and incubated for 1hr at 37°C. The blocking buffer was removed, serially diluted serum was added, and plates were incubated at 37°C for 1hr. After washing three times with wash buffer, secondary antibody [IgG(1:250)/IgG1(1:6000)/IgG2c (1:20000) in Wash buffer; Sigma Aldrich] was added and incubated for 1hr at 37°C. The plates were washed three times with wash buffer and reactions were developed with 3,3',5,5'- tetramethylbenzidine (TMB) for 20

minutes. The reaction was stopped using 1N HCl. Absorbance was measured at 450nm using the ELISA plate reader. The endpoint titer is determined by calculating the cut-off value as three times the background absorbance.

Anti-Influenza HA stalk Avidity ELISA:

Enzyme-linked immunosorbent assay plates (Flat bottom, Microlon 96-well high-binding plates manufactured by Greiner Bio-One) were coated with 100 ng of purified A/California/04/09 H1N1 virus protein in a carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing with wash buffer (1x PBS + 0.1% Tween-20), the plates were blocked with 10% dry milk in wash buffer and incubated for 1hr at 37°C. The blocking buffer was removed, serum was plated in quadruplets at 1:50 dilution (in wash buffer) and incubated for 1hr at 37°C. Ammonium thiocyanate (Sigma) at 1M concentration was added to the serum in duplicate, with control wells receiving wash buffer only. After 15 minutes, the samples were washed eight times with a wash buffer. IgG secondary antibody (1:250 in Wash buffer; Sigma Aldrich) was added and incubated for 1hr at 37°C. The plates were washed three times with wash buffer and reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB) for 20 minutes. The reaction was stopped using 1N HCl. Absorbance was measured at 450nm using a plate reader and final ratios were obtained by dividing normalizing samples with ammonium thiocyanate to corresponding ones without it.

Passive serum transfer experiment:

Adult male and female (8-10 weeks old) C57BL6 mice were vaccinated with the chimeric hemagglutinin based universal influenza vaccine regimen (Krammer et al.,

2013). Serum was collected 21 days post third vaccination. Serum from adult mice of each sex was pooled and an ELISA was performed to evaluate the average serum IgG (1:250 in Wash buffer; Sigma Aldrich) titer before the passive transfer. Unvaccinated male and female aged (68-70 weeks old) mice were injected 300uL of serum or PBS, with male and female aged mice receiving male and female adult, vaccinated pooled serum, respectively. Mice were allowed to rest for 2 hours post serum transfer, blood was collected from the recipient mice and ELISA was performed to check the antibody titer post-transfer. Mice were then challenged with 10^2 $TCID_{50}$ ma2009 H1N1 viruses and monitored for 14 days morbidity and mortality.

$TCID_{50}$ virus titration and quantification:

Lungs were homogenized in a serum-free DMEM media using PowerGen 125 Fisher Scientific homogenizer. Lung homogenates were spun at 4000 rpm for 20 minutes. The supernatant from each sample was collected and stored at -80ºC. For virus titration, lung homogenates were 10-fold serially diluted in DMEM (serum-free) and transferred to the 96 well cell-culture plates (Greiner Bio-one) containing confluent MDCK cells and incubated for 6 days at 32 ºC followed by fixation with 4% formaldehyde solution and staining with naphthol blue-black solution. Virus titers were calculated using the $TCID_{50}$ calculator(REED & MUENCH, 1938).

Hemagglutinin Inhibition Assay (HAI):

Sample preparation: Mouse serum samples were heat-inactivated for 35 minutes at 56°C. Samples (25µl) were diluted with 25µl of 5% sodium citrate and 50µl sterile normal saline solution (sodium chloride solution) and incubated at 37°C for 30 minutes. One

drop (50µl) of packed turkey red blood cells was added to the sample, mixed and incubated at 4°C for 30 minutes. Samples were centrifuged at 2000rpm for 10 minutes at 4°C and supernatant was separated.

Hemagglutination assay (HA): Hemagglutination assay (HA) was performed by serially diluting 25µl of the virus across a round-bottom 96 well plate in 25µl of 0.01M PBS. 50µl of 0.5% RBC suspension was added to the virus dilutions and the plate was sealed with a clear film and incubated at room temperature for 1 hour for HA reaction to develop. The HA titer was determined by the highest dilution causing complete agglutination.

Hemagglutination inhibition assay (HAI): For the hemagglutination inhibition assay (HAI), mouse serum was serially diluted across the plate in PBS to obtain 1:10 dilution. To 25µl of serum, an equal amount (25µl) of 4 HA units of virus dilution was added per well and incubated at room temperature for 1 hour. 50µl of 0.5% RBC suspension was added and the plate was sealed and incubated at room temperature for 2 hours for the mixture to agglutinate. The HAI titer was calculated as the reciprocal of the highest dilution causing complete inhibition of agglutination.

Statistical analyses:

Antibody responses and virus titers were analyzed by two-way ANOVA with age and sex as comparing factors and comparisons were performed using the Tukey multiple comparison test. Morbidity data (% change in body weight) were analyzed with two-way ANOVA (Mixed effects model) with Geisser-Greenhouse correction and comparisons were performed using the Tukey multiple comparison test. Data was considered

statistically significant at $p<0.05$. Statistics were performed in GraphPad Prism 8.0.1 software.

RESULTS

Age differences, driven mainly by females, were observed in stalk-specific antibody responses to H1N1 virus after vaccination

To explore the impact of biological sex and age on chimeric hemagglutinin (cHA)-based universal influenza vaccine immunity, adult (8-10 weeks) and aged (68-70 weeks) male (dark or light blue, respectively) and female (dark or light pink, respectively) mice were vaccinated thrice with chimeric hemagglutinin (cHA)-based UIV at 3-weeks interval and serum samples were collected on day 63 (i.e. 21 days after 3rd vaccination) (**Fig 3A**). The HA chimera used in the vaccine formulation is based on the backbone of H1 stalk. Hence, the antibody responses were checked against the H1N1 virus. The measured ELISA antibodies are stalk specific as there is a mismatch between the head region of coating reagents (H1 head) and head region of the chimeras (H9, H11, H12) used for immunization. HAI assay was performed, and we observed no result in all the serum samples tested, reconfirming the antibody response was HA-stalk directed not towards the HA head (data not shown). Stalk-specific anti-2009 H1N1 IgG (**Fig 3B;** *$p<0.05$**);** anti-2009 H1N1 IgG2c (**Fig 3C;** *$p<0.05$**);** anti-2009 H1N1 IgG1 (**Fig 3D**) responses measured indicated that adult mice generated a significantly higher anti-stalk IgG and IgG2c antibody response than the aged mice, whereas the IgG1 response had no significant difference. The mean antibody titers were lower for anti-HA stalk IgG1 than IgG and IgG2c antibodies, indicating lower induction of IgG1 in C57BL6 mice post influenza vaccination, as observed with the IIV as well (Fink et al., 2018).

Moreover, the age-associated decline in antibody responses was mainly driven by females, observed with a significant difference in total IgG and IgG2c antibodies between adult females and aged females, characterized with a significant age-associated decline in females specifically (**Fig 3B, C;** *$p<0.05$), which has also been observed previously with the IIV (Potluri et al., 2019). Antibody responses between the sexes, however, were similar in adult as well as aged mice.

To test if there is a difference in the avidity of the stalk specific antibodies between the cohorts' immune response, we performed the anti-H1N1 specific IgG antibody avidity ELISA on serum collected at 63 days post-vaccination. Avidity gives a measure of the overall strength of an antibody-antigen complex. It is dependent on the affinity of the antibody for the epitope along with the valency of both the antibody and antigen (Pullen, Fitzgerald, & Hosking, 1986). We observed that the adult mice had a significantly higher avidity response than the aged mice, indicating that the antigen-antibody binding is better in adult mice as compared to the aged mice (**Fig 3E;** *$p<0.05$**)**. Consistent with the total anti-stalk-specific IgG and IgG2c antibody responses, the age-related decline in antibody avidity was only observed among females, as we saw a significant difference in adult females and aged female mice (**Fig 3E;** *$p<0.05$**)**. Overall, these results indicate that not just the quantity but also the quality of antibody response post-vaccination with the cHA based UIV is significantly better in adult mice as compared to the aged mice and there is a salient sex-dependent effect of aging observed in immunity, driven by the females.

Antibody responses were analyzed by two-way ANOVA with age and sex as comparing factors and comparisons were performed using the Tukey multiple comparison test. Data was considered statistically significant at $p<0.05$.

Vaccination provided protection to both young and aged male and female mice as compared to their naïve counterparts after challenge.

Challenge studies dictating the efficacy of the cHA based UIV were performed in 7-8 weeks old females BALB/c mice. We wanted to expand on these results and test whether the protection stays consistent across different sex, age and strain of mice as compared to their unvaccinated counterparts. To test this, adult and aged mice, vaccinated or unvaccinated, were challenged with 10^2 $TCID_{50}$ of ma2009 H1N1 influenza A virus delivered intranasally **(Fig 4A).** Vaccinated mice were challenged 70 days post-vaccination and unvaccinated mice were age-matched with vaccinated mice during the challenge. One set of mice were euthanized at 5 days post-challenge to measure viral replication in the lungs and another set was monitored for 14 days post-challenge to evaluate morbidity (**Fig 4A**). Compared to unvaccinated mice, vaccinated mice had significantly lower virus replication in the lungs in males and females of the adult as well as the aged group. In adult mice, vaccinated male and female mice had significantly lower lung viral titers at 5 days post-challenge than their unvaccinated counterparts (**Fig 4B**; *$p<0.05$).

Similarly, in aged mice, vaccinated male and female mice were able to clear the virus better from the lungs, as evidenced by significantly lower viral titers than their unvaccinated counterparts (**Fig 4D**; *$p<0.05$). To evaluate morbidity, the percent change in body mass was measured daily for 14 days post-challenge, as a decline in body weight post-infection is one of the clinical symptoms exhibited in the mouse model. The unvaccinated males and females of both adult and aged categories suffered higher

morbidity than the vaccinated mice (**Fig 4C, E;** *$p<0.05$). Moreover, we confirmed the greater morbidity females experience as compared to the males after infection with the influenza virus, as evidenced by a significant difference in loss of body weight between unvaccinated females and unvaccinated males of both adult as well as aged mice (**Fig 4C, E**; *$p<0.05$). Comprehensively this indicated that vaccination protected mice of all sexes and ages better than the unvaccinated.

We also compared virus clearance from lungs and morbidity between the vaccinated adult and aged mice. There was a significant difference observed between the lung viral clearance between adult mice and aged mice **(Fig 5A; *$p < 0.05$).** The aged females had significantly higher lung viral titers than its adult counterpart, along with having the highest viral titers overall amongst all the cohorts **(Fig 5A).** Morbidity comparison between the four groups revealed that the aged females suffered the most morbidity, aged males experienced intermediate bodyweight loss and adult male and female mice had comparable morbidity (**Fig 5B**). Both the lung viral titers and morbidity studies correspond with the antibody data **(Fig 3 B, C, D)** in terms of demonstrating age-associated sex differences, where the vaccinated aged females had lowest antibody titers compared to the other vaccinated groups. These results overall suggest that vaccinated mice are better protected than unvaccinated mice. Meanwhile, amongst vaccinated, the vaccine-induced response is lowest amongst aged females and correspond in terms of protection with providing insufficient protection as compared to vaccinated aged males and adult mice.

Virus titers were analyzed by two-way ANOVA with age and sex as comparing factors and comparisons were performed using the Tukey multiple comparison test. Morbidity

data (% change in body weight) were analyzed with two-way ANOVA (Mixed effects model) with Geisser-Greenhouse correction and comparisons were performed using the Tukey multiple comparison test. Data was considered statistically significant at $p<0.05$

Female-biased effect of aging in antibody response is conserved across different subtypes of group 1 viruses but not group 2 influenza A viruses

Characterizing the universal nature of the stalk specific antibodies, Krammer et al. demonstrated that vaccination in adult female BALB/c mice and adult female fitch ferrets with the chimeric hemagglutinin (cHA)-based UIV offered protection against challenge with multiple group 1 influenza viruses including H5N1, H1N1, and H6N1 viruses (Krammer et al., 2013). To evaluate if host factors impact this cross-protection across multiple influenza subtypes, we vaccinated adult and aged mice three times at a 3-weeks interval with chimeric hemagglutinin (cHA)-based UIV and serum samples were collected 21 days post 3rd vaccination **(Fig 3A).** Serum IgG antibody response against H9N2 virus (HA and NA from A/Chicken/Hong Kong/G9/1997, backbone from A/PR/8/1934) (**Fig 6A)**; H6N3 virus (HA and NA from A/Swine/Missouri/4296424/2006, backbone from A/PR8/1934) (**Fig 6B**); H5N1 virus (HA and NA from A/Vietnam/1204/2004, backbone from A/PR8/1934) **(Fig 6C)**; and H3N2 virus (A/Hong Kong/1968) **(Fig 6D)** was evaluated in serum. The age-associated female bias was conserved across multiple group 1 influenza viruses, specifically H9N2 and H6N3. Total IgG response against H9N2 revealed a similar trend as observed with against H1N1 virus **(Fig 3B),** where adult mice generated a significantly greater antibody response than the aged mice, and aged females had the lowest titers as compared to the

other groups **(Fig 6A;** *$p < 0.05$**).** Similar responses were recorded against the H6N3 virus **(Fig 6B;** *$p < 0.05$**).** There were no significant sex or age differences in response to the H5N1 virus in the serum. However, aged females still had the lowest average IgG antibody titer (Adult males=703; Adult females=523; Aged males=286; Aged females=123) than other groups **(Fig 6C).** There was a minimum or undetectable response to the H3N2 virus **(Fig 6D)**, a Group 2 influenza virus, which reconfirms the results previously observed in mice studies (Krammer et al., 2013). Taken together, this data suggests that even the cross-reactive nature of the antibody response demonstrates differential response amongst the age groups and continues to decline significantly more in females.

Antibody responses were analyzed by two-way ANOVA with age and sex as comparing factors and comparisons were performed using the Tukey multiple comparison test. Data was considered statistically significant at $p<0.05$

Passive serum transfer of stalk specific antibodies from vaccinated adult mice does not protect against morbidity in sex-matched aged mice

Restating the results previously observed where the adult mice induced significantly higher antibody response than the aged mice, we wanted to test whether transferring these stalk specific antibodies produced in vaccinated adult mice would be protective in aged mice. We collected blood from adult male (Dark blue; solid) and female mice (Dark pink; solid) at 63 days post-primary vaccination with the chimeric hemagglutinin (cHA)-based UIV and tested the pooled serum for total IgG titers from both males and females. Both adult male and females had an equivalent endpoint titer of 6400 (Data not shown). Either serum or PBS was transferred from adult male and female mice into their aged

counterparts (light blue or pink; Open) (**Fig 7A**). After 2 hours of serum transfer, blood was collected again, and serum was tested to ensure successful serum transfer prior to challenge (Data not shown). Challenge with 10^2 $TCID_{50}$ of ma2009 H1N1 virus demonstrated that the aged males, as well as females receiving serum from adult females, suffered morbidity evidenced through the loss of body weight in the mice (**Fig 7B, C**). The passive serum recipient aged females, however, lost more body weight as compared to the serum recipient aged males. When compared with the vaccinated aged male and female mice, the passive serum recipient mice lost more body weight (**Fig 7B, C**). A transfer of serum from vaccinated adult females into naïve adult female mice has shown to confer protection from multiple group 1 viruses, whereas this passive transfer of antibodies is not enough to protect the aged mice sufficiently, and observed protection still is worse in females than males. This data overall indicates that there are underlying fundamental mechanisms of aging that exist differentially between the sexes and drive female-biased effects in aging.

Morbidity data (% change in body weight) were analyzed with two-way ANOVA (Mixed effects model) with Geisser-Greenhouse correction and comparisons were performed using the Tukey multiple comparison test. Data was considered statistically significant at $p<0.05$.

Figure 3: **Anti H1N1 stalk specific antibody response in vaccinated mice**

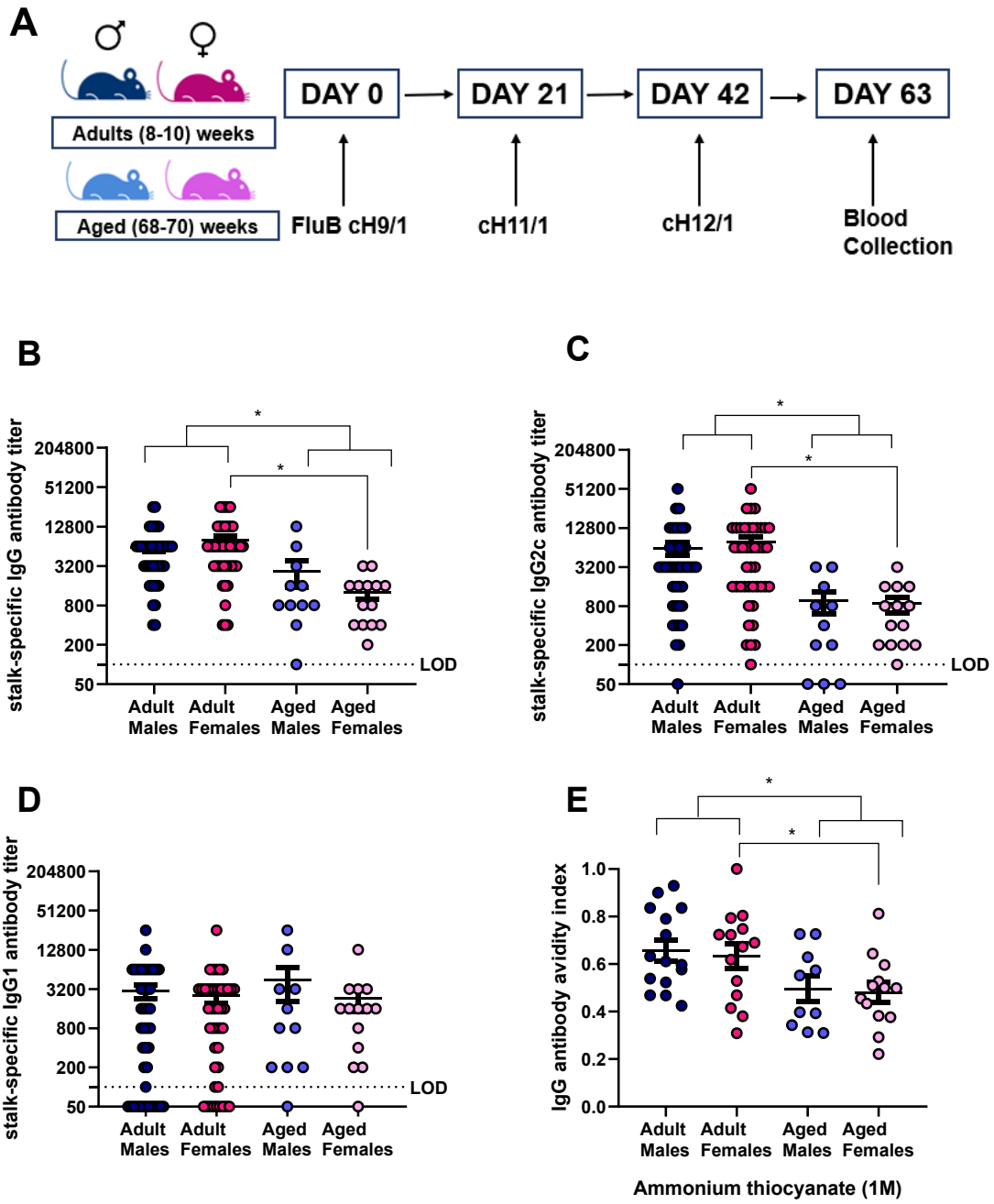


Figure 4: Lung viral titers and morbidity measure in vaccinated and unvaccinated mice post-challenge with the mouse-adapted 2009 H1N1 virus

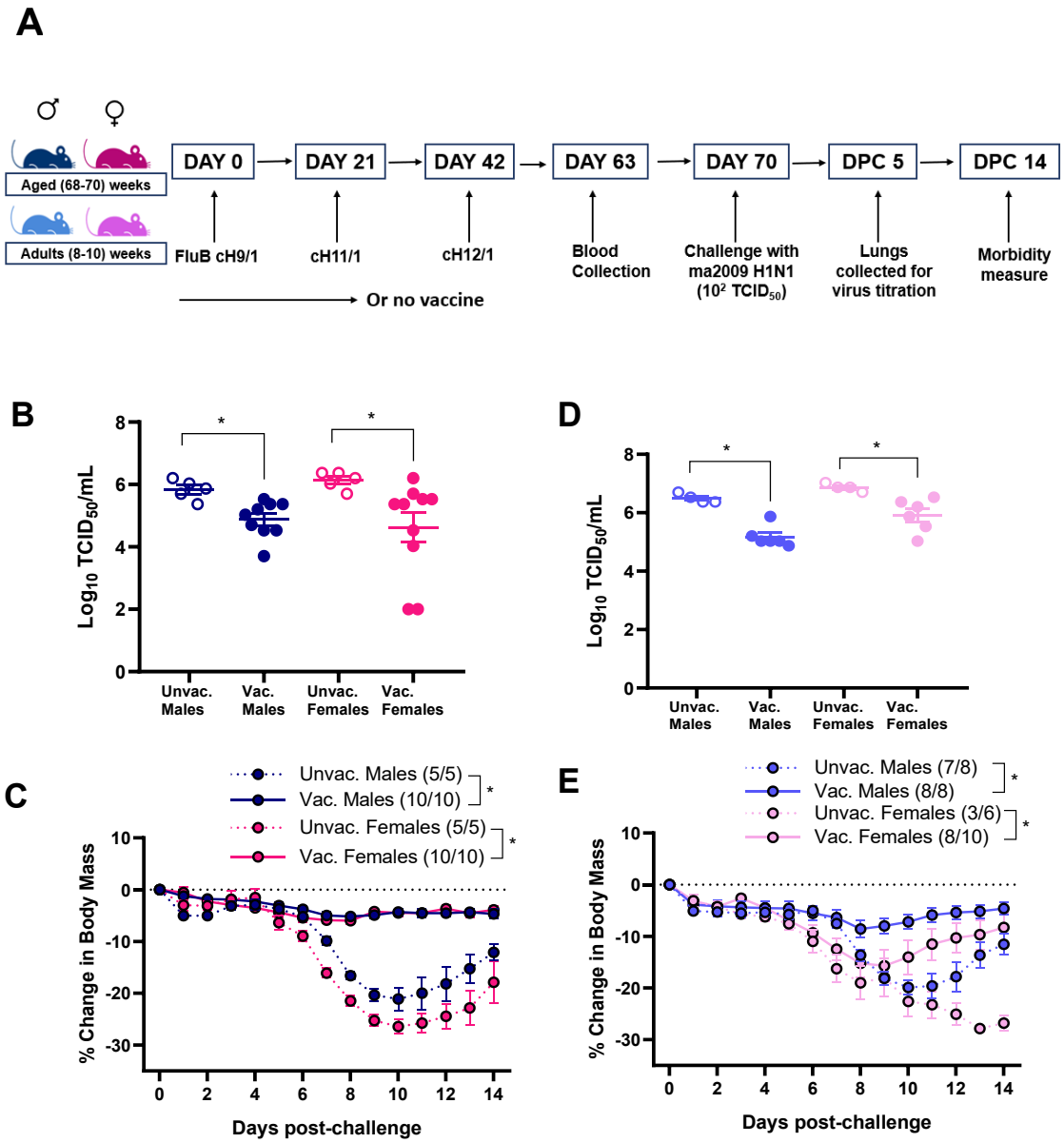


Figure 5: Comparison of lung viral titers and morbidity measure between adult and aged vaccinated mice post-challenge with the H1N1 virus

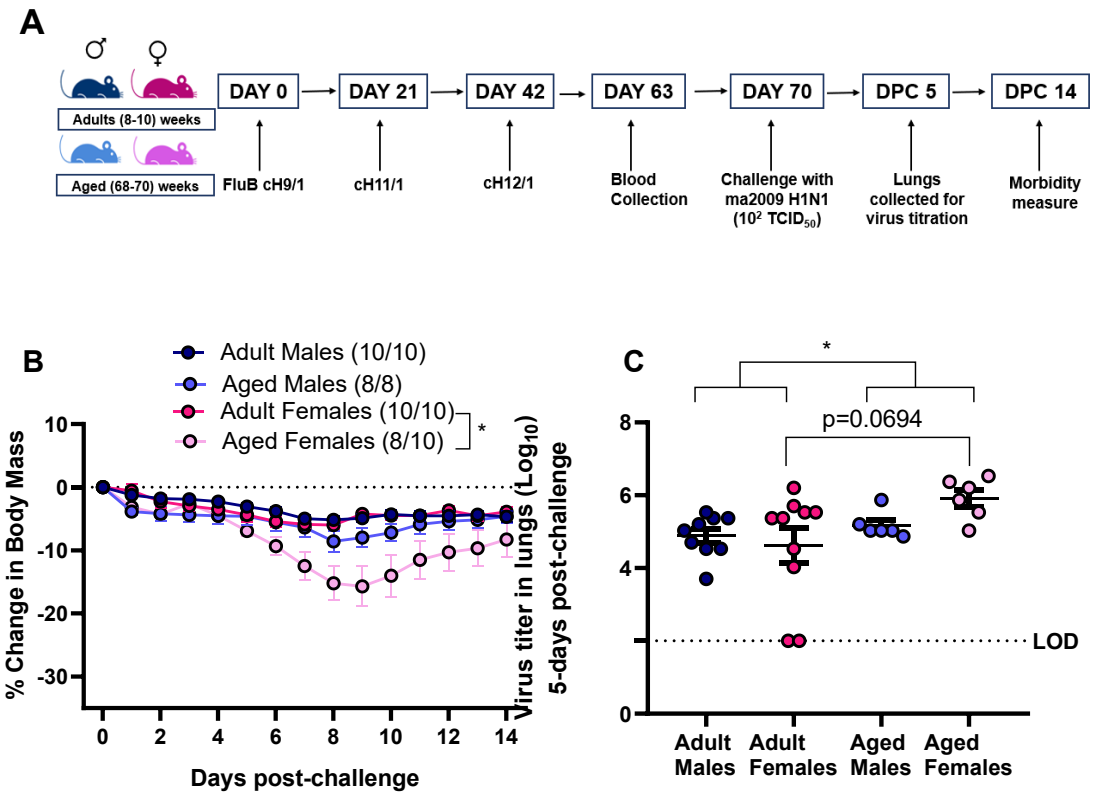


Figure 6: Heterosubtypic immune response post-vaccination with the chimeric hemagglutinin (cHA) based universal influenza vaccine

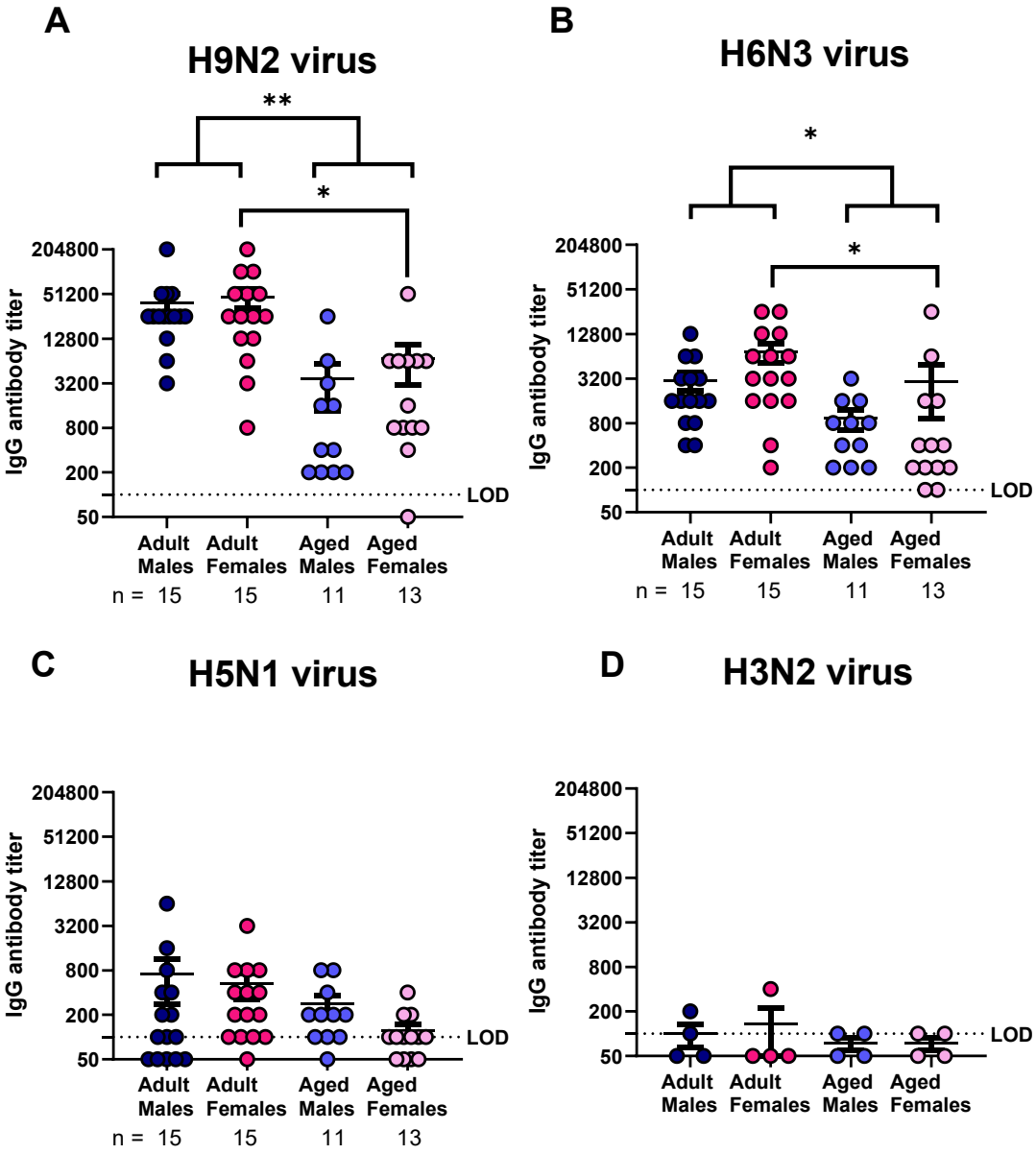
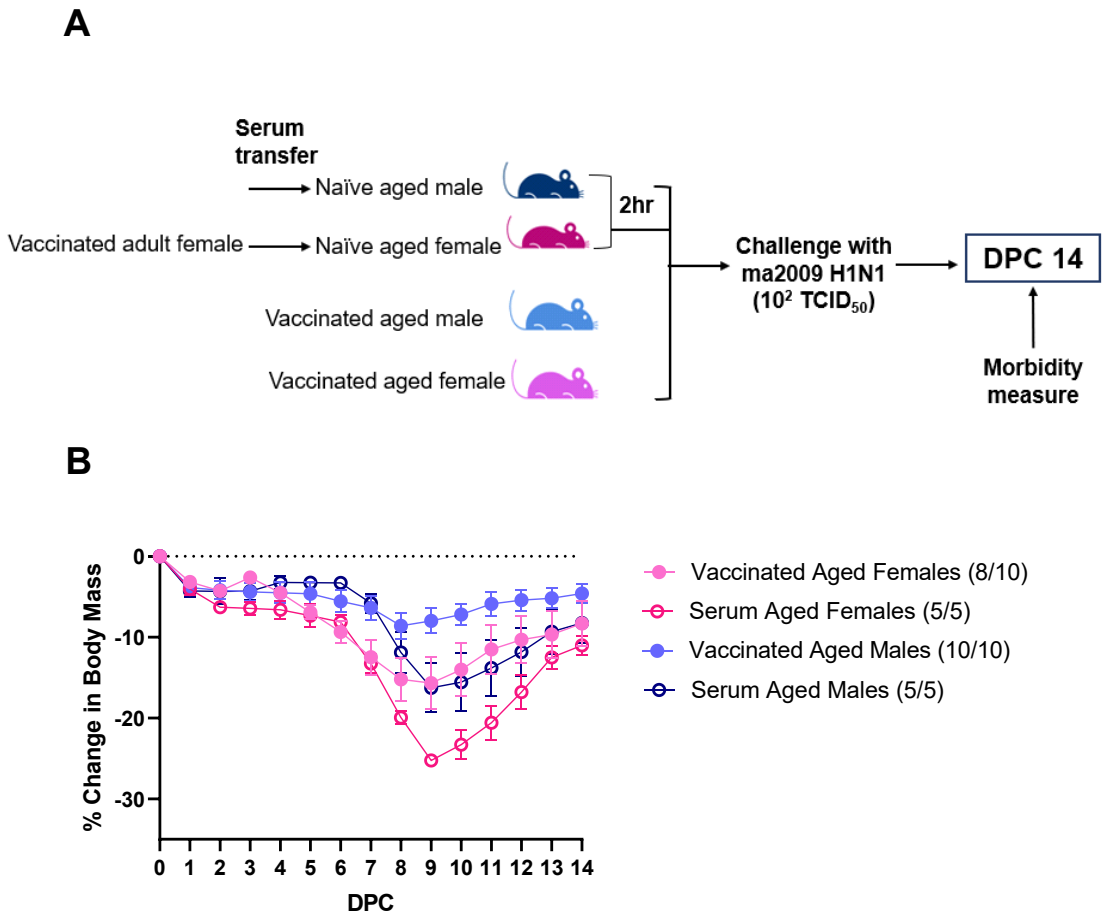


Figure 7: Comparison between protection conferred by the vaccine and passively transferred stalk-specific antibodies in aged mice



DISCUSSION

Influenza causes approximately 9 to 45 million illnesses and 12,000 to 61,000 deaths annually in the past decade in the United States alone (CDC: Disease Burden of Influenza). A 2018 study estimated the average annual economic burden of influenza to be around $11.2 billion (Putri, Muscatello, Stockwell, & Newall, 2018) in the United States. Moreover, seasonal influenza vaccines, which are the best line of defense against influenza transmission and infection, are still not optimized. Seasonal influenza vaccine manufacture requires annual update via rigorous surveillance and often results in limited vaccination coverage (Houser and Subbarao, 2015). The seasonal IIVs still produce a substantial health benefit in terms of the number of cases averting illnesses, hospitalizations, and clinic visits (Kostova et al., 2013). However, the vaccine efficacy varies vastly between different ages of the vaccinees each year and is affected by several viral as well as host factors (Dhakal & Klein, 2019) (Harding & Heaton, 2018) (States et al., 2020).

With the limitations observed with the seasonal influenza vaccines and advancement in influenza vaccine research, multiple platforms are coming into the picture. In terms of providing broader protection, pan-subtype, pan-group/multi-lineage and universal influenza vaccines are the key players in influenza vaccine research (Y. H. Jang & Seong, 2019). UIVs targeting the stalk region of the hemagglutinin (HA) protein, including the headless HA model, chimeric HA model, mosaic HA model and virus-like particles (VLP) expressing HA stalk domain are prospective platforms that can eventually enter commercialization. Recognizing the impact of host factors on seasonal influenza vaccine (primarily HA head specific response), this study explores the effect of

biological sex and age in the context of universal influenza vaccine (stalk specific) induced responses in mice models.

We focused on the cHA based UIV, which has been shown to protect female BALB/c mice and ferrets against challenge with multiple group 1 influenza viruses (Krammer & Palese, 2013). Humoral immunity is the critical mechanism of the protection conferred by the cHA-based universal influenza vaccination (Krammer et al., 2013). Hence antibody analysis is essential in our study to determine the quality and extent of protection provided by this vaccine regimen.

Although the HAI assay has been the gold standard as a relative measure of protection for head-specific antibody response, the cHA-based UIV induces a stalk-based response, which is non-neutralizing and hence cannot be detected by HAI assay (data not shown). The IgG antibody is a significant component of humoral immunity and is involved heavily in the ADCC mechanism during viral infection (Teillaud, 2012). In our study, the HA stalk-specific total IgG antibody response was observed to be significantly higher in the adult mice as compared to the aged mice against influenza A H1N1 virus. Moreover, this age difference was sex-specific, with aged females having a greater reduction in immune responses than the aged males. The age-associated decline in females was observed with total IgG2c antibodies as well**.** However, there was no significant difference in the IgG1 antibody response. A similar trend in IgG isotype response was observed post-vaccination with IIV in mice (Potluri et al., 2019). Our results align with the prior research demonstrating that the IgG2a/c antibody is the predominant immunoglobulin isotype in most viral infections in mice (Raval et al., 2012), which could be driving all observed differences.

Our study also highlights the cross-reactive nature of the stalk specific antibodies through a sex and age perspective. Adult mice exhibited significantly higher IgG titers against the H9N2 and H6N3 viruses as compared to aged mice, meanwhile, aged females demonstrated the lowest titers. No significant difference was observed in IgG response to the H5N1 virus and the overall titers were lower than those induced in response to other group 1 viruses**.** There was minimum detection of IgG response against the H3N2 virus, a group 2 influenza virus. Thus, our results suggest that this cHA based UIV platform creates a heterosubtypic response only against group 1 influenza viruses. A similar cHA based UIV based on the backbone of H3 stalk protects mice against group 2 influenza viruses. Perhaps, combining formulations of both aforementioned regimens could provide us with an improved UIV platform. The stalk-based antibody avidity is observed to be superior to the head-specific antibody response following pandemic H1N1 vaccination (Tete et al., 2016). The stalk specific antibody avidity, however, has not been evaluated between different age groups and sexes. We found the quality of antibody response to be higher in adult mice as compared to the aged mice, indicating an overall superior antibody response in adult mice.

Vaccination with a cHA-based universal influenza vaccine protects adult female BALB/c mice better than unvaccinated mice (Krammer et al., 2013). We confirmed this result in adult and aged, male and female C57BL6 mice using a sublethal dose of H1N1 influenza virus**.** Amongst vaccinated groups, challenge with H1N1 virus resulted in the highest morbidity in aged females, evidenced by the increased loss in body weight and higher viral replication in lungs, compared to other groups. Dendritic cells have shown to exhibit age-associated decreases in TLR3 protein expression (Shaw et al., 2011), which

could also be one of the reasons why there is insufficient protection in aged mice as this vaccine platform utilizes a Poly(I:C) adjuvant, a TLR3 stimulant. Future analysis using a higher dose of the Poly(I:C) adjuvant in aged mice can confirm this speculation.

There was no significant sex difference in immunity and protection in adult mice. Women show higher antibody responses, almost two-fold than men, following a full dose of TIV (Engler et al., 2008). Vaccination with IIV in mice models has demonstrated a sex difference in IgG, IgG2c and neutralizing antibody responses between males and females. Escape in X chromosome inactivation leading to a greater expression of toll-like receptor 7 (*Tlr7*) in B cells of females is associated with a higher antibody response post-vaccination with the IIV, than their adult male counterparts (Fink et al., 2018). The impact of sex hormones and genetic factors have not been explored in the context of stalk-based responses and can be crucial in understanding the sex-specific differences, or lack thereof, in adults following this vaccine regimen. Whether the introduction of a TLR7 agonist adjuvant such as Imiquimod in the vaccine formulation of this regimen can induce a differential immune response between the sexes can be potentially explored in the future.

Passive serum transfer of stalk specific antibodies from vaccinated young adult female mice resulted in protection in adult female mice (Krammer et al., 2013). Whether this protection persists across ages is also unknown. Passive serum transfer to aged mice did not significantly decrease morbidity in either males or females**.** Vaccinated aged mice exhibited a lower decline in body weight, compared to the passive serum recipients. As mentioned earlier, this could be due to a lack of induction of TLR3 pathway by Poly(I:C) adjuvant in passive serum recipient mice. Moreover, unvaccinated aged females receiving

serum from adult females suffered higher morbidity compared to their male counterparts, despite similar IgG antibody titers in the donor serum of males and females. Again, this reiterates the presence of a sex-based decline in immunity post-vaccination or infection and suggests the presence of fundamentally distinct mechanisms of aging between males and females.

Aging, as a phenomenon, is often assumed to be the same in both the sexes. However, lifespan, age-related diseases, infection susceptibility, human peripheral blood mononuclear cell (PBMC) analysis and our data indicate otherwise (Márquez et al., 2020). There is a sexual dimorphism observed in the life expectancy of humans where females are exceeding men, a phenomenon also observed in other mammalian species, including laboratory rats (Sampathkumar et al., 2020)(Clutton-Brock & Isvaran, 2007). Characterization of PBMCs in the human population of varying ages revealed an epigenomic indication of declining B cell loci, T cell population and increasing monocyte and cytotoxic cell functions at a higher magnitude in males (Márquez et al., 2020). Additionally, age-associated depletion of sex hormones (testosterone in men and estrogen and progesterone in women) has been linked to dysregulation of the immune system (Gubbels Bupp et al., 2018). Specific to influenza vaccination responses in humans, females have been linked with having a stimulatory effect of estrogen and males with a suppressive effect of testosterone on vaccine-induced antibody responses. It is evidenced with females of reproductive age having high neutralizing antibody seroconversion than aged females, post-vaccination with the IIV (Potluri et al., 2019), which also aligns with the responses we observed in this study. Investigating the role of sex hormones in the

context of universal influenza vaccines may help us get a better idea about the interplay between the HA-stalk specific response and the host factors.

The mechanism of action for cHA-based UIVs is the production of stalk-specific antibodies that mediate antibody-dependent cellular cytotoxicity (ADCC) (Krammer & Palese, 2013). Whether there are differences in ADCC response post-vaccination is still under investigation. In case of measles, lower sex-specific survival rates have been linked to decreased ADCC activity in adult females, post-infection and vaccination, than males (Atabani et al., 2000). Along similar lines, HIV infected men have shown to generate higher ADCC levels than females (Nag et al., 2004). Hence, more research towards influenza specific ADCC response is crucial in understanding the differences observed in immunity.

Another universal influenza vaccine candidate, utilizing a recombinant adenovirus expressing influenza A nucleoprotein (A/NP) and matrix 2 (M2) (A/NP + M2-rAd), revealed a decline in antigen-specific systemic antibody and T cell responses (García et al., 2016). The phase 1 clinical trial interim results of the chimeric HA-based UIV regimens with an adjuvanted chimeric haemagglutinin-based IIV demonstrated high serum IgG stalk-reactive antibody titers protective against all group 1 haemagglutinin-expressing viruses and no safety concerns post-vaccination (Bernstein et al., 2020). With the phase 1 clinical trial showing promising results, it is an excellent opportunity to take advantage of prior research done on host factors and explore their roles in vaccine before it enters the next step of production.

Aged individuals currently makeup around 962 million in the global population and are estimated to outnumber adolescents and youths by the year 2050 and can impact

population and economic dynamics globally (United Nations: World Population Ageing, 2017). They remain a high-risk population for various viral infections, including the current ongoing CoVid19 pandemic (CDC: Coronavirus Diseases 2020). With the dramatic differences observed in the immune response to influenza vaccination and infection in different ages and sexes of the population, it is vital to examine these factors for any upcoming candidate vaccine for their ability to protect different demographics within the population being vaccinated, particularly the elderly , prior to commercialization.

FIGURE LEGENDS

- **Figure 3: Anti H1N1 stalk specific antibody response in vaccinated mice**

  Adult (8-10 weeks) and aged (68-70 weeks) male (dark or light blue, respectively) and female (dark or light pink, respectively) mice were vaccinated thrice with chimeric hemagglutinin (cHA)-based universal influenza vaccine at 3-weeks interval and serum samples were collected on day 63 (i.e. 21 days after 3rd vaccination) (A). Stalk-specific anti-2009 H1N1 IgG (B); anti-2009 H1N1 IgG2c (C); anti-2009 H1N1 IgG1 (D); and anti-2009 IgG antibody avidity (E) responses were measured in serum. LOD represents limit of detection. Data represent mean ± standard error of the mean from two or three independent replications (n = 11-45/group) and significant differences between groups are denoted by asterisks (*$p<0.05$) based on two-way ANOVAs.

- **Figure 4: Lung viral titers and morbidity measured in vaccinated and unvaccinated mice post-challenge with the mouse-adapted 2009 H1N1 virus**

  Adult (8-10 weeks) and aged (68-70 weeks) male (dark or light blue, respectively) and female (dark or light pink, respectively) mice were either unvaccinated or vaccinated thrice with chimeric hemagglutinin (cHA)-based universal influenza vaccine at 3-weeks interval, serum samples were collected on day 63 and mice were challenged with 10^2 $TCID_{50}$ of ma2009 H1N1 influenza A virus at day 70 (A). To evaluate morbidity, the percent change in body mass were measured daily for 14 days post challenge in adults (B) and aged (D) mice (n = 5-10/group). Lung virus titers were measured on day 5 post challenge (n = 4-10/group) in adult (C) and aged (E) mice. LOD represents limit of detection. Data represent mean ± standard error of the mean

from two independent replications (n = 4-10/group) and significant differences between groups are denoted by asterisks (*p<0.05) based on repeated measures ANOVA for morbidity data and unpaired T-test for virus titer.

- **Figure 5: Comparison of lung viral titers and morbidity in adult and aged vaccinated mice post-challenge with the H1N1 virus**

  Adult (8-10 weeks) and aged (68-70 weeks) male (dark or light blue, respectively) and female (dark or light pink, respectively) mice were vaccinated thrice with chimeric hemagglutinin (cHA)-based universal influenza vaccine at 3-weeks interval, serum samples were collected on day 63 and mice were challenged with 10^2 $TCID_{50}$ of ma2009 H1N1 influenza A virus on day 70 (A). To evaluate morbidity, the percent change in body mass was measured daily for 14 days post challenge (B). Lung virus titers were measured on day 5 post challenge (n = 6-10/group) (C). Data represent mean ± standard error of the mean from two independent replications (n = 4-10/group) and significant differences between groups are denoted by asterisks (*p < 0.05) based on repeated measures ANOVA for morbidity data and two-way ANOVA for virus titer.

- **Figure 6: Heterosubtypic immune responses post-vaccination with the chimeric hemagglutinin (cHA) based universal influenza vaccine**

  Adult (8-10 weeks) and aged (68-70 weeks) male (dark or light blue, respectively) and female (dark or light pink, respectively) mice were vaccinated thrice with chimeric hemagglutinin (cHA)-based universal influenza vaccine at 3-weeks interval and serum samples were collected on day 63 (i.e. 21 days after 3rd vaccination). Stalk-specific IgG antibody response against (A) H9N2 virus (HA and NA from A/Chicken/Hong

Kong/G9/1997, backbone from A/PR/8/1934); (B) H6N3 virus (HA and NA from A/Swine/Missouri/4296424/2006, backbone from A/PR8/1934); (C) H5N1 virus (HA and NA from A/Vietnam/1204/2004, backbone from A/PR8/1934) and (D) H3N2 virus (A/Hong Kong/1968). LOD represents limit of detection. Data represent mean ± standard error of the mean from two independent replications (n=11-15/group) and significant differences between groups are denoted by asterisks (*p<0.05) based on two-way ANOVA.

- **Figure 7: Comparison between protection conferred by the vaccine and passively transferred stalk-specific antibodies in aged mice**

  Aged (68-70 weeks) vaccinated and unvaccinated males (Solid or Open light blue, respectively) and female (Solid or Open light pink, respectively) are age matched prior to the experiment. Unvaccinated male receive serum from adult vaccinated mice (not denoted) and unvaccinated female mice receive serum from adult unvaccinated mice (not denoted). Blood is collected following passive transfer of serum from the sexes. Both vaccinated and unvaccinated mice are challenged with 10^2 $TCID_{50}$ of ma2009 H1N1 influenza A virus (A). To evaluate morbidity, the percent change in body mass was measured daily for 14 days post challenge (B). Data represent mean ± standard error of the mean from one experiment (n = 5-10/group) and significant differences between groups are denoted by asterisks (*p<0.05) based on two-way ANOVAs.

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EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Expected May 2020
Master of Science (ScM), Molecular Microbiology and Immunology
CGPA: 3.47/4

Vellore Institute of Technology, Vellore, TN, India
July 2014 - July 2018
Bachelor of Technology, Biotechnology
CGPA: 3.97/4

SKILLS

Virology: Virus culture (BSL-2 facility); Virus Propagation; Virus titration by $TCID_{50}$
Immunology: ELISA; Virus Neutralization assay; B-cell Isolation; FACS analysis
Cellular Biology: Mammalian cell culture; Cell Transfection; Cell Media Preparation
Molecular Biology: DNA/RNA Isolation; q-PCR; SDS-PAGE
Animal Handling: Small Animal (Mouse) handling; Retro-orbital bleeding; Intramuscular, intraperitoneal and intranasal injection/inoculation; Euthanization and sample collection; Isolation of cells from spleen; Preparation of lung homogenates
Software: Microsoft Office Excel; PowerPoint; GraphPad Prism; C++(Basic)
Languages: English – Fluent; Hindi – Fluent; Marathi – Fluent; Spanish – Basic

RELEVANT EXPERIENCE

Research Assistant, Immunology Research
November 8 – May 2020
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA
Advisor: Dr. Sabra L. Klein
Thesis: Evaluating the impact of biological sex and aging on universal influenza vaccine induced immunity in mice

- Design, plan and execute *in vivo* vaccine experiments in C57BL/6 mouse model
- Immunize adult and aged mice with universal flu vaccine
- Collect blood, lungs and spleen samples from mice for immunological analysis
- Perform virus titration, neutralizing antibody assay and ELISA using mouse

samples
- Perform neutralizing antibody assay on influenza vaccinated/infected human sera
- Maintain mammalian cell line, prepare lab reagents, maintain lab equipment

Insectary Technician
May 2019 – May 2020
Johns Hopkins Malaria Research Institute, Baltimore, MD, USA
- Organization and cleaning of trays used for mosquito egg hatching and larval growth

Research Assistant, Gene and Cell therapy Research
December 2017 – April 2018
Vellore Institute of Technology, Vellore, India
Advisor: Dr. Everette Jacob Remington
Thesis: Generation of CD80 gene knockout cell line using CRISPR-Cas9
- Transfection of HEK cell line for the production of recombinant viruses
- Testing the knockout efficiency of different guide RNA sequences

In-Plant Trainee
Wockhardt Research Centre, Aurangabad, India
June 2017 – July 2017
- Used molecular biology techniques to analyze bacterial cell samples
- Optimize techniques involved in identification and extraction of protein and nucleic acids

.

RELEVANT COURSEWORK

Fundamental Virology; Immunology and Infectious Diseases; Molecular Biology; Microbiology; Vaccine development and applications; Biological basis of vaccine development; Basic Epidemiology; Stem cell technology

ACHIEVEMENTS

Publications:
- Sabra L. Klein, Santosh Dhakal, Rebecca L. Ursin, **Sharvari Deshpande**, Kathryn Sandberg, and Franck Mauvais-Jarvis. *Biological sex impacts COVID-19 outcomes*. Accepted in PLOS Pathogen, April 22, 2020.

- Potluri, T., Fink, A.L., Sylvia, K.E., Dhakal, S., Vermillion, M.S., vom Steeg, L., **Deshpande, S**., Narasimhan, H. and Klein, S.L., 2019. *Age-associated changes in the impact of sex steroids on influenza vaccine responses in males and females*. NPJ vaccines, 4(1), p.29

- **Deshpande, S**., Singh, S., Panneerselvam, A. and Rajeswari, V.D., 2019. *Nutrients in Caffeinated Beverages—An Overview. In Caffeinated and Cocoa Based Beverages* (pp. 367-389). Woodhead Publishing

Posters:

- **Sharvari Deshpande**, Santosh Dhakal, Sabra L. Klein. *Sex differences in antibody responses and protection following receipt of a monovalent inactivated influenza vaccine in mice*. Poster presentation at Vaccine Day (April 2019), Johns Hopkins Vaccine Initiative, Johns Hopkins Bloomberg School of Public Health.

Certificates:

- Animal Care and Use Certificate, Johns Hopkins University
  November 2018

Scholarships/Awards:

- MTS scholarship, Johns Hopkins Bloomberg School of Public Health
  September 2019
- Second Prize, Bioinspired Design Summer Fest (BIDSF), VIT India.
  April 2017
  Topic: "Development of Early Diagnostic Tool-kit to Detect Oral Cancer Using a Chewing Gum"

Leadership & Volunteering

- Events head: Creativity Club, VIT, Vellore India
  May 2016 – May 2017
- Volunteer: Living Classroom Foundation
  October 2018